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Treatment with high dose of atorvastatin reduces vascular injury in diabetic rats

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ABSTRACT

Background: Previous reports showed conflicting results regarding the treatment effects of statin on Diabetes mellitus (DM). We investigated how treatment with high dose of atorvastatin affects the impaired vascular function in diabetic rats.
Methods: Atorvastatin (80 mg/kg/day, oral gavage, 4 weeks) or its vehicle was administered to male control or streptozotocin (STZ)-induced diabetic rats. Aortic segments were used to investigate the vascular reactivity, protein expression of cyclooxygenase-2 (COX-2) and nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) 1 (NOX1) and superoxide anions levels.
Results: Atorvastatin treatment did not affect glycemia levels. In diabetic rats, the vascular reactivity to phenylephrine increased compared with controls and the atorvastatin treatment reduced this response. Removal of the endothelium increased the response to phenylephrine in control rats, but not in the diabetic group. Atorvastatin increased the endothelial modulation in diabetic rats. L-NAME (100 μM) increased the reactivity in all groups, but this effect was greater in atorvastatin-treated diabetic rats. Indomethacin (10 μM) and NS398 (1 μM) decreased the contractile response in diabetic rats and atorvastatin reversed these effects, without changing COX-2 expression. Apocynin (30 μM) decreased the phenylephrine response in diabetic rats, which also showed increased NOX1 and superoxide anions; these effects were prevented by atorvastatin treatment.
Conclusions: The results suggest that treatment with high dose of atorvastatin, independent of glycemia, improves endothelial function in aortas from diabetic rats by reducing the constrictor prostanoids derived from COX-2 and by reducing the oxidative stress by NADPH oxidase, as well as a possible increasing of nitric oxide participation.

Introduction

Diabetes mellitus (DM) is a known risk factor for developing cardiovascular disease and its subsequent complications. The endothelial damage in diabetes, a major cause of cardiovascular complications, independent of other cardiovascular risk factors, include hyperglycemia, systemic inflammation and increased oxidative stress [1]. A number of mechanisms have been suggested to account for endothelial dysfunction in DM, including an increase in cyclooxygenase constrictor prostanoids [2–4], deficiency of the nitric oxide synthase (NOS) co-factor tetrahydrobiopterin (BH4) [1,5] and an increase in oxidative stress [1,6]. Previous reports have demonstrated that in type 1 diabetes, nitric oxide (NO)-mediated relaxation is actually increased at an early stage and that impaired relaxation occurs only as the disease progresses [7].

As mentioned above, DM increases the reactive oxygen species (ROS) levels, and these free radicals play a role in the pathogenesis of DM that is associated with vascular complications [8]. The increase of ROS in DM may result from reduced activity of antioxidant enzymes, such as superoxide dismutase (SOD) [9] and catalase [10], and an increase in NADPH oxidase activity [6,11,12]. Therefore, the increase in oxidative stress observed in DM may lead to a reduction in NO bioavailability and accelerate atherosclerosis in conductance vessels [13].

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Cholesterol-lowering drugs, such as 3-hydroxy-3-methylglutaryl (HMG)-coenzyme (CoA) reductase inhibitors (statins), are vasoprotective in DM. The mechanism by which statins are vasoprotective may involve the upregulation of endothelial NOS expression and NO production [14] and the reduction of thromboxane A2 (TXA2) and superoxide anion generation [15,16]. In fact, low doses of atorvastatin (20–50 mg/kg/day) have been demonstrated to inhibit NADPH oxidase, which contributes to the reduction of superoxide anion generation in aortic segments from both hypertensive and diabetic rats [16–18].

High-dose atorvastatin (80 mg/kg) may also have a beneficial effect, as previous studies have shown that it can reduce the incidence of peripheral artery disease to a greater extent than can the typical dose of simvastatin used in statin therapy (20–40 mg/kg) [19]. In contrast, previous reports have demonstrated that high doses of atorvastatin (80 mg/kg) impair endothelium-dependent microvascular function in patients with type 1 DM [20]. Thus, these studies have shown conflicting results regarding the treatment effects of statins on the diabetic rats vascular function. Therefore, we tested the hypothesis that a high dose of atorvastatin (80 mg/kg) promotes additional beneficial effects in the vascular function of conductance vessels in diabetic rats.

Material and methods

Experimental animals

Male Wistar rats weighing 220–250 g were used. All experiments were conducted in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (n°. 8023, revised 1978) and approved by the local Ethics Committee on Animal Use. The rats had free access to water and were fed standard chow *ad libitum*.

Animals were randomly allocated to four groups: (1) untreated control (Ct, atorvastatin vehicle, carboxymethylcellulose 0.5%); (2) atorvastatin-treated control (Ct + At, atorvastatin 80 mg/kg/day); (3) untreated streptozotocin (STZ)-induced (50 mg/kg) diabetic (Db, carboxymethylcellulose); (4) atorvastatin-treated diabetic (Db + At, atorvastatin 80 mg/kg/day). All treatments took 4 weeks, daily, by oral gavage.

DM type 1 was induced and established as previously described [2]. The blood glucose levels, glycemic response curve and insulin resistance were measured using a hemoglucotest (ACCU-CHEK® Active analyzer, Roche, Mannheim, Germany).

After treatment, the rats were anesthetized with pentobarbital (35 mg/kg, intraperitoneal) and killed by exsanguination. The thoracic aortas were carefully dissected and divided into 4–5 mm cylindrical segments. To analyze the COX-2 and NOX-1 protein expression and evaluate superoxide anions production these arteries were frozen at −80 °C until the day of experiment.

Vascular reactivity measurements

Segments of thoracic aorta were mounted in isolated tissue chambers as previously described [21]. After the smooth muscle and endothelial integrity tests, increasing concentrations of phenylephrine (0.1 nM to 30 mM) were applied to get the concentration–response curve.

The influence of the endothelium on the response to phenylephrine was investigated after the endothelium has been mechanically removed [21]. This was confirmed by the inability of 10 mM acetylcholine to produce relaxation (represented by E^{-}).

The effects of the following drugs were evaluated: a non-selective NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME, 100 μM), a non-selective cyclooxygenase inhibitor (indomethacin, 10 μM), a COX-2 inhibitor (NS398, 1 μM) and a general antioxidant (apocynin, 30 μM). These drugs were added 30 min prior to the generation of the phenylephrine concentration–response curves.

Western blot analysis

Proteins from homogenized arteries were quantified as previously described [22]. Were used mouse monoclonal antibodies anti-COX-2 (1:200; Cayman Chemical, MI, USA) or anti-NADPH subunit NOX1 (gp^{22phox}, 1:1000; Transduction Laboratories, Lexington, UK), and anti-mouse immunoglobulin antibody conjugated to horseradish peroxidase (1:5000; StressGen, Victoria, Canada). The same membrane was used to determine α-actin expression using a mouse monoclonal antibody (1:5000; Sigma) as a loading control.

In situ *detection of vascular superoxide anions*

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate superoxide anions production *in situ*, as previously described [23]. The mean fluorescence densities in the target region were calculated using the Image J software (Wayne Rasband, Bethesda, MD, USA).

Statistical analyses

Vasoconstrictor responses induced by phenylephrine were normalized expressed as the percentage of the tone generated by 75 mM KCl. For each concentration–response curve, the maximum effect (Emax) and the concentration of agonist that produced one-half of Emax, the sensitivity (pD2), were calculated using nonlinear regression analysis in GraphPad Prism Software 6 (San Diego, CA, USA).

The results are expressed as mean ± SEM (Standart Error of the Mean) and "*n*" indicates the number of rats used. The variables were tested by test of normality Kolmogorov–Smirnov. Then, differences were analyzed using Student's *t*-test or one-way analyses of variance (ANOVA), followed Tukey *post-test*. Differences were considered statistically significant at $p < 0.05$. Two-sided p was used.

Drugs and reagents

Were used crystalline atorvastatin calcium (Morepen Laboratories, New Delhi, India), l-phenylephrine hydrochloride, L-NAME, indomethacin, acetylcholine chloride, sodium thiopental, apocynin (Sigma) and NS398 (Cayman Chemical, MI, USA). Atorvastatin was dissolved in carboxymethylcellulose and all other drugs were dissolved in distilled water. Other salts and reagents were purchased from Sigma and Merck (Darmstadt, Germany).

Results

Atorvastatin treatment does not affect body weight or hyperglycemia

At baseline, there was no difference in body weight among the four treatment groups (Ct: 248 ± 14 g, $n = 19$; Ct + At: 226 ± 12 g, $n = 17$; Db: 237 ± 20 g, $n = 33$; and Db + At: 235 ± 28 g, $n = 24$; $p > 0.05$). However, after treatment, both of the diabetic groups lost weight (Ct: 324 ± 26 g; Ct + At: 317 ± 30 g; Db: 233 ± 49 g*; and Db + At: 244 ± 61 g*; *$p < 0.05$). The diabetic rats also exhibited severe hyperglycemia at 1 and 4 weeks after STZ administration (Table 1). Atorvastatin treatment did not affect hyperglycemia (Table 1), glycemic curve (Table 2) or insulin resistance test (Table 3).